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Nox1 activation by βPix and the role of Ser-340 phosphorylation

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ABSTRACT

Rac is an activating factor for Nox1, an O_2^--generating NADPH oxidase, expressed in the colon and other tissues. Rac requires a GDP-GTP exchange factor for activation. Nox1 activation by βPix has been demonstrated in cell lines. We examined the effects of βPix and its phosphomimetic mutant on endogenous Nox1 in Caco-2 cells transfected with Noxo1 and Noxa1. βPix expression enhanced O_2^- production in resting cells and cells stimulated with EGF or phorbol ester. βPix(S340E) further enhanced O_2^- production, while βPix(S340A) eliminated the βPix effect. βPix(S340E), but not βPix(S340A), had higher affinity and GEF activity for Rac than wild-type βPix. These results suggest that βPix phosphorylation at Ser-340 upregulates Nox1 through Rac activation, confirming Rac as a trigger for acute Nox1-dependent ROS production.

Structured summary of protein interactions:
Rac binds to **betaPix** by pull down (View interaction)
Rac-GTP physically interacts with **PAK-PBD** by pull down (View interaction)

1. Introduction

Nox1 is an enzyme that generates superoxide (O_2^-) and belongs to the NADPH oxidase (Nox) family [1]. Nox1 is expressed in the colon, vascular system, and other organs, and is thought to be involved in several cell functions such as proliferation, vascular contraction, host defense, and cytoskeletal remodeling [2]. The enzyme is an integral membrane protein complexed with $p22^{phox}$ and requires a small GTPase Rac as well as Noxa1 and Noxo1 for its activation [3–5]. Cheng et al. hypothesized that Rac is a major trigger that can acutely activate Nox1-dependent ROS production [3]. Noxo1 was reported to be autoinhibited by self-masking through an intramolecular interaction [6,7], and the masking was recently shown to be released by phosphorylation [8,9]. It has also been suggested that Noxa1 is suppressed by an intramolecular interaction, although the exact mechanism is unclear [10,11].

It is known that Rac in the cytosol is complexed with rhoGDI in the GDP-bound form and requires GDP/GTP exchange for activation and membrane transfer. Therefore, a guanine nucleotide exchange factor (GEF) protein must be involved in the Nox1 activation pathway. βPix is one of the GEFs for rho families such as Rac, and belongs to the diffuse B-cell lymphoma (DBL) family [12]. Previously, Park et al. [13] demonstrated that βPix is involved in Nox1 activation in epidermal growth factor (EGF)-stimulated cell lines.

Regarding βPix regulation, many studies have been performed and a number of activation mechanisms have been postulated, as for other DBL family members [12], which include phosphorylation, dimerization, and binding of phosphoinositide or protein factors such as p21-activated protein kinase (PAK) or 14-3-3 protein [14]. Despite these studies, the mechanism for βPix activation remains ambiguous.

Phosphorylation of βPix in response to EGF was systematically analyzed in HEK293 cells using mass spectrometric analysis [15]. Multiple phosphorylation sites were observed in βPix, including Ser-340. Phosphorylation of Ser-340 was also observed in EGF-stimulated HeLa cells [16]. However, the relationship between βPix phosphorylation and its activation has not been studied.

Caco-2 cells are derived from epithelial colon cancer cells and express Nox1 and $p22^{phox}$ intrinsically as well as Rac and βPix [13]. In the present study, to clarify the relationship between phosphorylation of βPix and its activation, we examined the effects of phosphomimetic and unphosphorylatable mutants of βPix on O_2^- production in Caco-2 cells. We found that substitution of Glu for Ser-340 of βPix enhanced O_2^- production in resting cells or cells stimulated with EGF or phorbol ester. To elucidate the mechanism

Abbreviations: Nox, NADPH oxidase; S340E, βPix(S340E); S340A, βPix(S340A); GEF, guanine nucleotide exchange factor; DBL, diffuse B-cell lymphoma; EGF, epidermal growth factor; PAK, p21-activated protein kinase; GppNHp, 2′(3′)-O-(N-methylanthraniloyl)-β:γ-imido guanosine 5′-triphosphate; PKA, protein kinase A; PKC, protein kinase C; PI 3-P, phosphatidylinositol 3-phosphate

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of these phenomena, we further examined the effects of these mutants on its Rac binding and GEF activity.

2. Materials and methods

2.1. Cells

Caco-2 cells (Riken BRC, Ibaraki, Japan) were cultured in Minimum Essential Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biowest, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin at 37 °C in 5% CO_2.

2.2. Reagents

EGF and bovine erythrocyte superoxide dismutase (SOD) were purchased from Sigma–Aldrich (St. Louis, MO). Diogenes was obtained from National Diagnostics (Atlanta, GA). PreScission Protease, glutathione-Sepharose (G-Sepharose), HiTrap CM FF, and HiTrap Q HP were purchased from GE Healthcare (Little Chalfont, UK). 2′(3′)-O-(N-methylanthraniloyl)-β:γ-imido guanosine 5′-triphosphate (Mant-GppNHp) was obtained from Biolog Life Science Institute (Bremen, Germany).

2.3. Plasmid construction

Human βPix cDNA (isoform a) was a generous gift from Dr. Takashi Nagase (Kazusa DNA Research Institute, Chiba, Japan). The βPix cDNA was amplified by PCR using 5′- and 3′-primers with attached *Bam*HI and *Eco*RI sites, respectively, and subcloned into the pEF-BOS vector [17] or pGEX-6P vector between the *Bam*HI and *Eco*RI sites. The vectors pEF-BOS-myc-Noxa1 and pEF-BOS-HA-Noxo1 were generous gifts from Dr. Hideki Sumimoto (Kyushu University, Fukuoka, Japan). pGEX-2T-Rac1 was a generous gift from Dr. Dave Lambeth (Emory University, Atlanta, GA). For mutation of βPix, the cDNA in the pEF-BOS-myc or pGEX-6P vector was mutated to convert Ser-340 to Glu or Ala using a QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by dideoxynucleotide-based sequencing.

2.4. Transfection

Cells (1.0×10^6) were washed with buffer T (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 m $MgCl_2$), suspended in the same buffer (0.4 ml), mixed with 5 μg of pEF-BOS-myc-βPix (or mutant), pEF-BOS-myc-Noxa1, and pEF-BOS-HA-Noxo1 in a pulse cuvette with a 2-mm gap (NEPA Gene, Chiba, Japan), and kept on ice for 10 min. The mixture was then pulsed with an ECM600 electroporator (BTX, San Diego, CA) at 950 V/cm from a 300-μF capacitor. After electroporation, the cells were cultured for 24 h in the presence of fetal bovine serum. The expression of each protein was verified by Western blotting.

2.5. Stimulation and measurement of O_2^-

The above-treated cells were suspended in buffer C (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, 17 mM HEPES pH 7.4) at 1×10^5 cells/ml. Subsequently BSA (0.03% final concentration) and 10 μl of modified Diogenes solution (diluted twice with phosphate-buffered saline (PBS) containing 50 mM luminol [18]) was added to the suspension. After incubation for 5 min at 37 °C, the cells were incubated for 5 min at 37 °C in the presence of EGF (in 5% trehalose) or vehicle. The luminescence was measured and accumulated for 1 min at 37 °C using an AB-2270 luminometer (Atto, Tokyo, Japan).

2.6. Preparation of βPix and its mutants

The pGEX-6P plasmid containing the βPix or mutant cDNA was transfected into *Escherichia coli* BL21 cells and expressed with 50 μM isopropyl thiogalactoside at 16 °C for 20 h. The cells were suspended in buffer S (150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.025% sodium deoxycholate, 50 mM Tris–HCl pH 8.0), and lysed by sonication at 60 W for 12×30 s. The lysate was mixed with G-Sepharose beads, and washed with PBS, 0.5% Triton X-100, and buffer K (50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM ATP, 1 mM dithiothreitol, 50 mM Tris–HCl pH 8.0) to dissociate DnaK. βPix was then released by digestion with PreScission Protease (60 U) at 4 °C for 4 h in buffer S containing 0.025% Tween-20 instead of sodium deoxycholate. The eluate was treated with 2 mM DIFP, dialyzed at 5 °C for 4 h against 20 mM Tris–HCl (pH 9.0) containing 150 mM NaCl and 0.025% Tween-20, and concentrated with an Amicon Ultra (30-kD cut-off). The samples were supplemented with leupeptin (40 μg/ml), aprotinin (10 μg/ml), and 2 mM DIFP, and applied to the HiTrap Q HP column (5 ml) on an AKTA prime system. Wild-type or mutant βPix was eluted with an NaCl gradient (150–300 mM) in 50 mM Tris–HCl buffer (pH 9.0). All samples from the column were dialyzed against 20 mM potassium phosphate buffer (pH 7.5) and concentrated with the Amicon Ultra.

2.7. Preparation of Rac, GST-Rac, and its mutant

Rac1(C189S) (Rac), GST-Rac1(C189S) (GST-Rac), and GST-Rac1(Q61L, C189S) (GST- RacQ61L) were expressed and purified as described [19]. Purification of the GST-proteins was performed according to the manufacturer's protocol (GE Healthcare).

2.8. Pull-down assay

Purified wild-type or mutant βPix (1 μM) was mixed with GST-Rac or GST-RacQ61L (1 μM) in 400 μl of 50 mM HEPES (pH 7.6) containing 150 mM NaCl and 0.1 mM EDTA, and incubated for 30 min at 5 °C. Next, 100 μl of G-Sepharose (50% slurry in the same buffer) was added to the mixture and the mixture was rotated at 4 °C for 30 min, followed by centrifugation at $800 \times g$ for 2 min at 5 °C. The supernatant (unbound) was removed and the precipitate (bound) was washed twice with 100 mM phosphate buffer (pH 7.0) containing 100 mM KCl and 0.1% Triton X-100 and once with 200 mM Tris–HCl (pH 8.5) containing 200 mM NaCl and 0.5% Triton X-100. The wild-type or mutant βPix was eluted with the same buffer containing 20 mM glutathione (eluate). Aliquots of the bound, unbound, and eluate fractions were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue. The band intensities of wild-type or mutant βPix in these fractions were quantified by imaging analysis with Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD) and the percentages of eluted βPix to total βPix were calculated according to the following equation: $\% I = I_{\text{eluted}}/(I_{\text{bound}} + I_{\text{unbound}}) \times 100$.

Endogenous Rac-GTP level was estimated by PAK-PBD pull-down assay using a Rac1 Activation Assay Biochem Kit (Cytoskeleton, Denver, CO) according to the manufacturer's protocol. The samples were subjected to Western blotting with anti-Rac1 monoclonal IgG and subsequently with peroxidase-conjugated goat anti-mouse IgG. The PVDF membrane was treated with ECL Prime Detection Reagent (GE Healthcare) and the bands were detected with a LAS-1000 luminescent image analyzer (Fujifilm, Tokyo, Japan). After soaking in a stripping buffer (Takara Bio, Shiga, Japan) the membrane was treated with rabbit anti-actin IgG (Sigma–Aldrich) and then goat anti-rabbit IgG (MP Biomedicals, Santa Ana, CA).

2.9. GEF assay

The assay mixture contained 0.5 μM βPix, 5 μM Rac, 2 μM Mant-GppNHp, 10 μM GDP, 10 μM $MgCl_2$, and 150 mM NaCl in 600 μl of 20 mM Tris–HCl (pH 7.5). The increase in fluorescence (excitation at 357 nm and emission at 443 nm) was monitored for 2 min in a spectrofluorometer (F-7000; Hitachi, Tokyo, Japan).

3. Results

3.1. O_2^- production in Caco-2 cells transfected with Noxa1 and Noxo1

First, we examined the O_2^- production in Caco-2 cells using Diogenes, which detects O_2^- and not H_2O_2. In the resting state, Caco-2 cells produced no O_2^-, and EGF treatment did not elicit any detectable amounts of O_2^- production (Fig. 1). After transfection of Noxo1 and Noxa1, activating factors for Nox1, a substantial amount of O_2^- was produced even in the resting cells, and EGF treatment further enhanced the production. Transfection of either Noxo1 or Noxa1 alone did not induce O_2^- production even in the stimulated cells (data not shown). When SOD was included in the system, luminescence was reduced to the basal level, confirming that the system only detected extracellular O_2^-.

3.2. Enhancement of O_2^- production in Caco-2 cells by βPix

Next, we examined the effect of βPix on O_2^- production in Caco-2 cells. When βPix was expressed by itself, practically no increase in luminescence was observed in the resting or EGF-stimulated cells (Fig. 1). Coexpression of Noxo1 and Noxa1 with βPix enhanced O_2^- production in the resting or EGF-stimulated cells by about 50%. These results are different from the report by Park et al. [13], who demonstrated that βPix expression by itself enhanced ROS production, although they used serum-deprived Caco-2 cells and a different probe that detects intracellular H_2O_2.

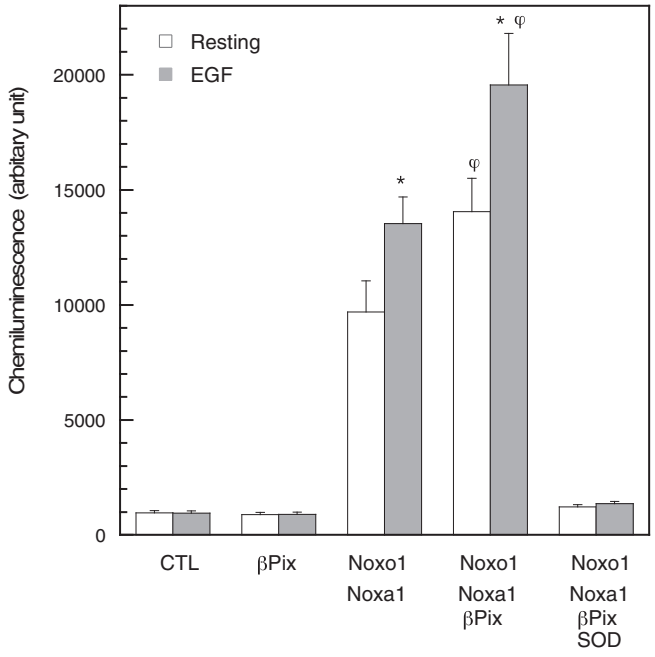

Fig. 1. O_2^- production in Caco-2 cells transfected with Noxa1 and Noxo1. Caco-2 cells were transfected with Noxa1, Noxo1, and/or βPix, and cultured for 24 h. The cells were suspended in buffer C and incubated with Diogenes for 5 min at 37 °C. Then the cells were incubated with EGF or vehicle for 5 min at 37 °C before luminescence measurement. In some experiments superoxide dismutase (SOD) was included in the system. *: $P < 0.05$ vs. the resting cells; ϕ: $P < 0.05$ vs. without βPix.

3.3. Effect of a phosphomimetic mutation at Ser-340 of βPix on O_2^- production

Mayhew et al. [15] showed that Ser-340 in the PH domain is phosphorylated by EGF treatment. Fig. 2 depicts the domain structure of human βPix and the sequence around this residue. The sequence is highly conserved in a variety of animals. To examine whether phosphorylation of Ser-340 influences the O_2^- production, we expressed a phosphomimetic or unphosphorylatable mutant of the residue in Caco-2 cells. Substitution of Glu for Ser-340 increased the O_2^- production by 48% in the resting cells (Fig. 3, Table 1), while replacement of Ser-340 by Ala markedly decreased the O_2^- production to the control level, which was much lower than the level for wild-type βPix, suggesting that Ser-340 was partially phosphorylated even in the resting cells. Substitution of Glu for Ser-340 also further enhanced the O_2^- production in the

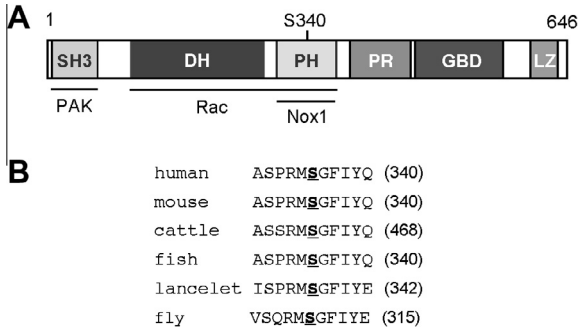

Fig. 2. Domain structure of human βPix and the site of Ser-340. (A) The domain structure of βPix and putative binding sites for PAK, Rac, and Nox1. SH3, Src homology-3; DH, Dbl homology; PH: pleckstrin homology; PR, proline-rich region; GBD, GIT1 binding domain; LZ, leucine zipper. (B) The sequence around Ser-340 of human βPix compared with the corresponding regions of other animals: human, isoform a (NP003890); mouse, isoform c (NP050098); bovine, isoform X1 (XP002692038); fish (zebrafish) isoform A (DQ656108); lancelet (XM 002593729); fly (*Drosophila melanogaster*) (AJ224676).

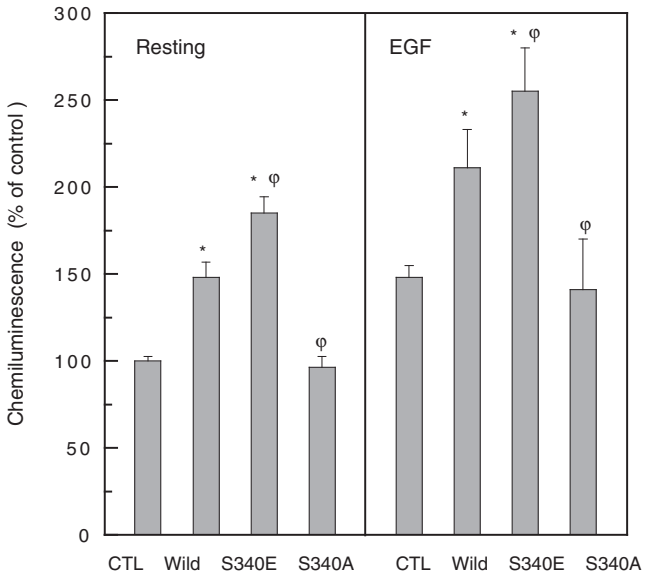

Fig. 3. Effect of phosphomimetic or unphosphorylatable mutations of βPix on O_2^- production. Cells transfected with myc (CTL), βPix (wild), or a mutant in addition to Noxa1 and Noxo1 were treated with or without EGF at 37 °C for 5 min, and subjected to luminescence assay. Values were obtained by subtraction of the basal luminescence from the observed one. The data are expressed as % luminescence of that with control of the resting cells. *: $P < 0.05$ vs. CTL; ϕ: $P < 0.05$ vs. wild.

Table 1
Effect of βPix or a mutant on O_2^- production induced by several agonists.

Agonist	% O_2^- production			
	CTL	βPix	S340E	S340A
None	100 ± 2.5 (1.00)	148 ± 8.8 (1.48)	185 ± 9.3 (1.85)	96.4 ± 6.3 (0.96)
EGF	148 ± 6.8 (1.00)	211 ± 22.0 (1.43)	255 ± 25.1 (1.72)	141 ± 29 (0.95)
PMA	660 ± 51.4 (1.00)	858 ± 72.3 (1.30)	1240 ± 62.7 (1.88)	640 ± 58.1 (0.97)

The cells were transfected with myc (CTL), βPix, S340E, or S340A and treated with buffer (None) for 5 min, EGF for 5 min, or PMA for 10 min. The values in parentheses are the ratios of O_2^- production to CTL with each agonist.

EGF-stimulated cells and substitution of Ala reduced the O_2^- production to the control level (Fig. 3).

3.4. Effects of the mutations of βPix on its Rac binding

As βPix(S340E) showed higher O_2^- production, we examined whether the Rac binding affinity was enhanced by the mutation. βPix and its mutants recombinantly expressed and purified (Fig. 4A) were used in pull-down assays with GST-Rac (GDP-bound) or GST- RacQ61L (GTP-bound) (Fig. 4B). The assays showed that βPix interacted with GST-Rac and less tightly with GST-RacQ61L (Fig. 4B and C). Furthermore, the S340E mutant showed a higher Rac binding affinity (2.3-fold with GDP-form), while S340A mutant had a similar affinity to wild-type βPix.

3.5. Effects of the mutations of βPix on its GEF activity

To clarify whether the phosphomimetic mutation of Ser-340 enhanced the GEF activity for Rac, the GEF activities of βPix and its mutant were measured with purified Rac and βPix using Mant-GppNHp, a fluorescent non-hydrolyzable GTP analog. In the absence of βPix, Rac underwent some GDP/GTP exchange at 10 μM $MgCl_2$ (Fig. 5, Table 2). When βPix was added to the mixture, the exchange rate was increased by 40%. The S340E substitution further increased the rate by 31%. These results show that phosphorylation of Ser-340 enhances the GEF activity of βPix.

3.6. Effects of βPix on O_2^- production in Caco-2 cells stimulated by other agonist

Phorbol 12-myristate 13-acetate (PMA) is an agonist that is often used to induce O_2^- production in cell lines. Therefore, we examined the effect of βPix on PMA-induced O_2^- production (Table 1). PMA enhanced the O_2^- production by 7-fold, and βPix increased the rate of O_2^- production by 43%. The S340E mutant further increased the O_2^- production, while the S340A substitution eliminated the βPix effect, similar to the case for EGF.

3.7. Effect of βPix on endogenous Rac activation

We estimated Rac-GTP level in the lysates from transfected cells using PAK-PBD pull-down assay (Fig. 6). Imaging analysis of the bands showed that βPix(S340E) expression increased Rac-GTP level by 66%, and the ratio to the total Rac amount was calculated to be 2.3-fold of that with the control.

4. Discussion

In the present study, we examined the O_2^- production in Caco-2 cells transfected with βPix and Nox1 activators and found the following points: (i) transfection of βPix alone did not induce O_2^- production; (ii) transfection of both Noxo1 and Noxa1 induced O_2^- production; (iii) EGF or phorbol ester did not induce O_2^- production, but did enhance it when Noxo1 and Noxa1 were overexpressed;

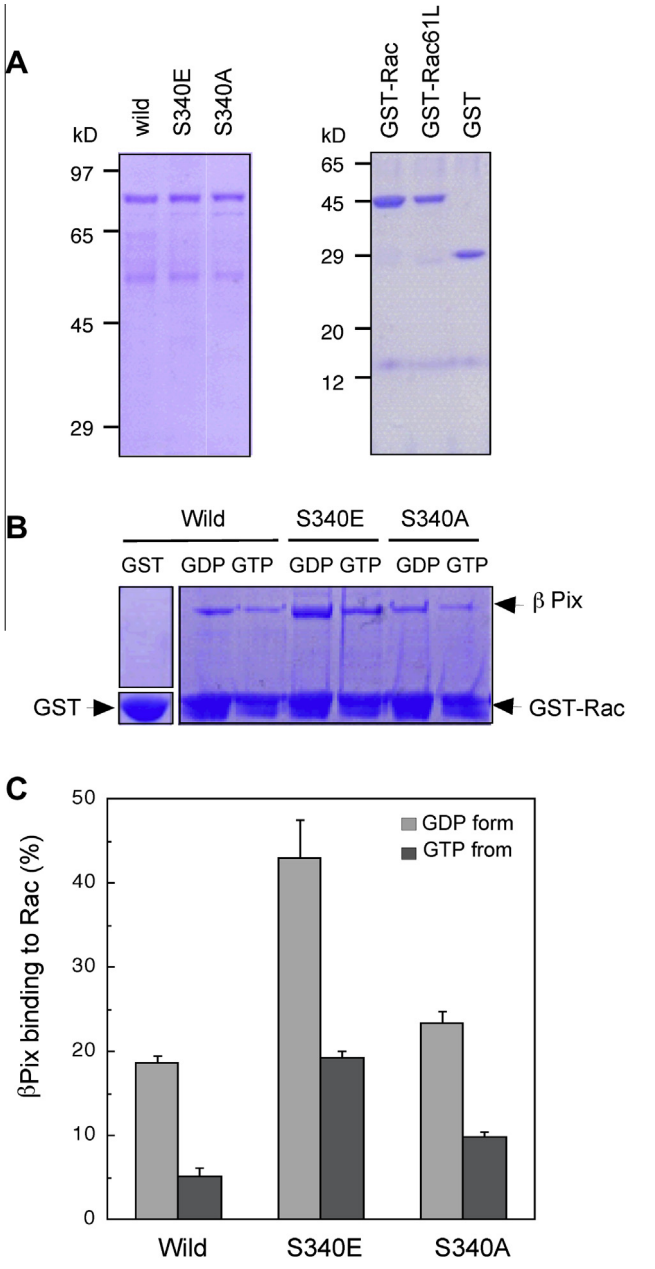

Fig. 4. Rac binding to βPix and the effects of βPix mutations. (A) SDS–PAGE of the purified βPix and its mutants along with GST-Rac and GST-RacQ61L. (B) Pull-down assay: βPix or a mutant was mixed with GST-Rac (GDP-form) (GDP), or GST-RacQ61L (GTP-form) (GTP) and incubated for 30 min at 5 °C. G-Sepharose was added to the mixture, incubated at 5 °C for 2 min, and centrifuged. The precipitates were washed and eluted with glutathione. Aliquots of the eluates were subjected to SDS–PAGE and stained with Coomasie Brilliant Blue. (C) The band intensity of βPix was quantified and its percentage of the total intensity of applied βPix was calculated. *: $P < 0.05$ vs. wild; $^{\Phi}$: $P < 0.05$ vs. GDP-form.

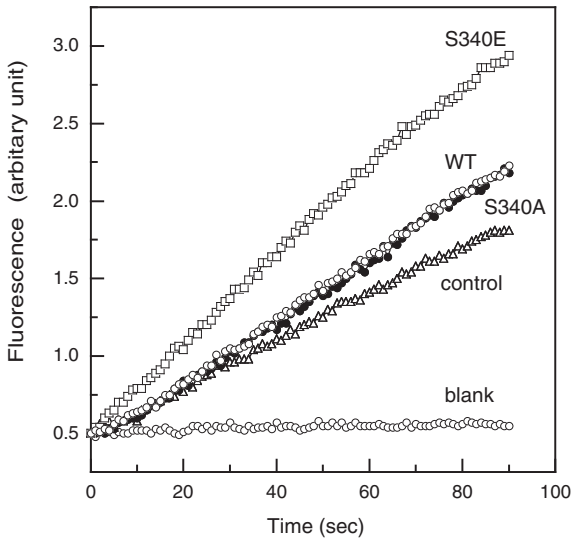

Fig. 5. Effect of Ser-340 mutation on its GEF activity. Assay mixture contained βPix (0.5 μM), 5 μM Rac, 2 μM Mant-GppNHp, 10 μM GDP, and 10 μM $MgCl_2$ in 20 mM Tris–HCl (pH7.5) containing 150 mM NaCl. The fluorescence was monitored for 2 min at 25 °C. Blank, Mant-GppNHp only; control, Rac plus Mant-GppNHp. The data is the typical one of three independent experiments.

Table 2
GEF activity of βPix and its mutants on Rac.

βPix	% GEF activity[a]
CTL	100 ± 1.8
Wild-type	140 ± 13[b] (1.00)
S340E	184 ± 27[c] (1.31)
S340A	144 ± 30[d] (1.03)

[a] The values in parentheses are the ratios of the activities to that with the wild-type.
[b] $P < 0.05$ vs. CTL.
[c] $P < 0.05$ vs. the wild-type.
[d] Not significant vs. wild-type.

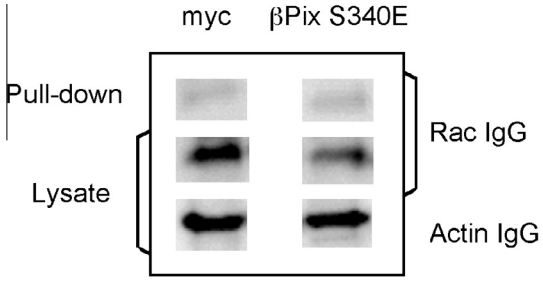

Fig. 6. Effect of βPix on the endogenous Rac activation. Caco-2 cells transfected with Noxa1, Noxo1, and βPix(S340E) or myc were lysed and subjected to pull-down assay with GST- PBD (PAK) on glutathione agarose. The lysates and pull-down fractions were subjected to Western blotting with anti-Rac IgG and/or anti-actin IgG.

and (iv) expression of βPix further enhanced O_2^- production in resting and stimulated cells.

Regarding the activation mechanism of βPix, several hypotheses have been presented. Phosphorylation is one of the possible mechanisms. Mayhew et al. [15] systematically studied the phosphorylation sites of transfected βPix in HEK293 cells, and found that several residues, including Ser-340, were phosphorylated in EGF-stimulated cells. However, the role of phosphorylation in βPix activation remains to be clarified.

In this study, we found that the S340E mutant upregulated Nox1 activation, while the S340A mutant eliminated the effect of βPix, suggesting that phosphorylation of Ser-340 enhances the ability of βPix to activate Rac. Pull-down assays with GST-Rac and βPix suggested that Ser-340 phosphorylation enhances the ability of Rac binding. In addition, it was found that the S340E mutant had higher GEF activity than wild-type βPix.

Ser-340 is located in the PH domain, which was postulated to interact with Nox1 (Fig. 2) [20]. It is plausible that phosphorylation of Ser-340 might enhance the interaction of βPix with Nox1. The PH domain was also found to bind phosphatidylinositol 3-phosphate (PI 3-P) or another phosphoinositide [13], although it remains unclear whether the binding enhances the catalytic activity of βPix. In some of the DBL family members, phosphoinositide binding enhances their translocation to the membrane. In our GEF assay, βPix activated Rac without PI 3-P, at least to some extent, and addition of PI-3P did not increase the GEF activity (data not shown).

One of the interesting findings in this study is that unstimulated Caco-2 cells produced a substantial amount of O_2^- when transfected with Noxa1 and Noxo1. Although Caco-2 cells express Rac endogenously, GDP/GTP exchange must occur for Nox1 activation. The present observations imply that a certain amount of Rac was already activated in the resting cells. In relation to this, a small amount of Rac-GTP has been observed in serum-treated Caco-2 cells [21,22]. On the other hand, it is not clear why transfection of Noxo1 and Noxa1 are required while they are expressed endogenously in colon cancer cells [23] as well or even more in normal colon cells [2].

Regarding the kinase involved in Ser-340 phosphorylation, NetphosK, a web-based prediction program, indicates protein kinase A (PKA) as a candidate for the sequence around Ser-340 (Fig. 2), but the score is not high. Recently, Rennefahrt [24] predicted PAK as a candidate based on a new algorithm of specificity profiling, and actually showed that PAK phosphorylates Ser-340 in transfected HEK293 cells. Indeed, it is known that PAK is activated in serum-treated or EGF-stimulated NIH3T3 cells [27]. PAK was originally assumed to be a downstream effecter of Rho GTPases, but recent studies showed that Rac sometimes functions in the downstream of PAK [25,26]. In spite of these, however, the kinase responsible for Ser-340 phosphorylation in Caco-2 cells is unclear at present. Further studies will be required to clarify this point.

In our system, PMA greatly enhanced O_2^- production in Caco-2 cells. PMA is a well-known protein kinase C (PKC) activator, and was recently found to induce Noxo1 phosphorylation, which unmasks Noxo1 to activate Nox1 [8,9]. PMA may activate other kinases, including PAK, through the Raf/MAPK cascade. In addition, it was demonstrated that PMA activates EGF receptors in place of EGF by increasing their autophosphorylation through an unknown mechanism [28,29]. These multiple activation mechanisms may explain the marked effect of PMA on Nox1 activation.

EGF has often been assumed to protect colon cells against reactive oxygen species-induced damage and maintain the intestinal barrier integrity [30]. However, the present data showed that when Noxo1 and Noxa1 were overexpressed, EGF rather enhanced O_2^- production in colon cancer cells. In this context, it is noteworthy that tumor necrosis factor-α induces Noxo1 expression in HT8 colon epithelial cells [31]. Although no agonists that induce Noxa1 expression have been identified, it is activated by Src [32], which relieves the autoinhibition of Noxa1 [10,11]. It is also known that expression of Noxa1 and Noxo1 is sometimes enhanced in colon cancer cells as mentioned above [23].

It has been shown that βPix is involved in cytoskeletal reorganization or migration in some types of cells [33,34]. Thus βPix expressed in normal colon epithelial cells [35] may also be involved in these cellular events. βPix expressed in cancerous colon

epithelial cells including Caco-2 was shown to be involved in cell migration and adhesion [36]. These events are often related to ROS production either in normal [37] or cancer cells [38] although the involvement of Nox1 in these events in Caco-2 cells is not clear at present.

In this study, we have shown that a phosphomimetic mutant of βPix enhanced O_2^- production, and suggest that phosphorylation of βPix may regulate Nox1 through Rac activation. This is the first example of regulation of Nox 1 through phosphorylation of a GEF.

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